

IMMUNOLOGICAL ENHANCEMENT AGENT COMPRISING N-TERMINAL

PEPTIDE OF p43 AS AN EFFECTIVE COMPONENT

FIELD OF THE INVENTION

The present invention relates to an immunological enhancement agent, more particularly, to an immunological enhancement agent comprising specific peptides having immunological activity as an effective component.

BACKGROUND OF THE INVENTION

Cells undergoing programmed cell-death (known as apoptosis) are rapidly removed by monocyte-derived macrophages. This phenomenon infers that apoptotic cells might secrete specific factors and these factors cause chemotaxis to leucocyte and monocyte. The endothelial monocyte-activating polypeptide II (hereinafter, referred to as "EMAP II") is known as one of the specific factors because it is released from the apoptotic cells and causes chemotaxis.

The EMAP II is the C-terminal domain of the p43 protein - the precursor of the EMAP II, consisting of 312 amino acids - and it is cleaved to form by caspase-7, in which aspartic acid - the 146th amino acid of p43 protein - is activated in apoptotic cells (Quevillon, S. *et al.*, *J. Biol. Chem.*, 272:32573-32579, 1997; Behrendorf, M. A. *et al.*, *FEBS Lett.*, 466:143-147, 2000). The structure and maturation of the EMAP II is similar to that of an IL-1 β , a cytokine involved in a proinflammatory response, and 14.5kDa of the IL-1 β is cleaved to form from 33kDa of inactive pre-IL-1 β by ICE(caspase-1). An EMAP II is the mediator of proinflammatory response that induces the expression of tissue factor, tumor necrosis factor (hereinafter, referred to as "TNF") and interleukin-8 (hereinafter, referred to as "IL-8") in mononuclear phagocyte and polymorphonuclear leucocytes. Also, in a tissue expressing a high level of EMAP II mRNA, macrophages are accumulated. This means that an EMAP II is a chemotaxis material leading macrophage to dead cells. It is known that the EMAP II acts as a cytokine, and the 15 amino acids of the N-terminal domain of an EMAP II play a vital role in the reaction (Quevillon, S. *et al.*, *J. Biol. Chem.*, 272:32573-32579, 1997; Kao, J. *et al.*, *J. Biol. Chem.*, 269:9774-7982, 1994; Kao, J. *et al.*, *J. Biol. Chem.*, 267:20239-20247, 1992; Kao, J. *et al.*, *J. Biol. Chem.*, 269:25106-25119, 1994; Knies, U. E. *et al.*, PNAS USA, 95:12322-12327, 1998). In the United States Patent No. 5,641,867, it is described that the N-terminal domain of an EMAP II that comprises arginine-isoleucine-glycine-arginine-isoleucine-threonine is an important residue in cytokine function of the EMAP II. Recently, it was

reported that the EMAP II repressed the growth of primary and metastatic tumor in proliferating endothelial cells that are not causing particular side-effect in normal cell (Schwarz, M. A. *et al.*, *J. Exp. Med.*, 190:341-353, 1999).

On the other hand, the p43 protein is expressed extensively. The expression level of p43 protein varies temporally and spatially, especially in a developing mouse. For example, it was shown that the expression of a p43 in the lung of mouse from 8 days to 16 days after its birth was increased dramatically. In addition, the p43 is highly expressed in the microglial cells in the lesions of autoimmune disease such as encephalomyelitis, neuritis and uveitis. The high expression level of the p43 in specific developmental stage and tissues suggests that the p43 could have unexpected functions in angiogenesis, inflammation, and apoptosis (Tas, M. P. R., and Marray, J. C., *Int. J. Biochem. Cell. Biol.*, 28:837-841, 1996; Schwarz, M. J. *et al.*, *Glia*, 20:365-372, 1997; Schuesner, H. J. *et al.*, *Glia*, 20:365-372, 1997; Berger, A. C. *et al.*, *J. Immunother.*, 23:519-527, 2000).

As mentioned previously, the EMAP II - C-terminal domain of p43 - has been studied extensively for its cytokine activities; however, p43 (i.e. pro-EMAP II) and its N-terminal domain have not been understood. Therefore, the present inventors have studied to disclose that a p43 can act as a more effective cytokine and as an immunological enhancement agent than the EMAP II. We did this by comparing cytokine activity of a p43 with that of an EMAP II, C-terminal domain of p43 in the PCT application No.PCT/KR00/00630.

The present inventors compared the secretion pattern of a p43 with that of an EMAP II in normal cells and apoptotic cells, and reported that the cytokine acting in physiological condition was the p43 and not the EMAP II. Since the EMAP II is secreted during the late stage of apoptosis in which cells are completely destroyed in normal cells, it is not active in the early stage of apoptosis; whereas, the p43 is constitutively secreted from various cells irrespectively of apoptosis (Ko YG et al., A cofactor of tRNA synthetase, p43, is secreted to up-regulate proinflammatory genes, *J. Biol. Chem.* 2001, Apr 5, 276).

While studying the p43, the present inventors achieved the present invention by discovering that the peptides, including N-terminal domain of p43, showed an excellent cytokine activity with the result of estimating cytokine activities of deletion-mutants of the p43 protein.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide an immunological enhancement agent that comprises the N-terminal domain of the p43 showing an excellent immunological activity as an effective component.

In order to accomplish the object above, the present invention provides an immunological enhancement agent that comprises a peptide having an amino acid sequence represented by SEQ ID NO:1 to SEQ ID NO:3 as an effective component.

According to the present invention, the p43(1-147), the p43(1-108) and the p43(91-256), which comprise the N-terminal domain of the p43 protein, can act as a cytokine to increase the amount of the TNF and the IL-8. As a result, they can improve an immune response and be used as an effective immunological enhancement agent.

BRIEF DESCRIPTION OF DRAWINGS

Fig. 1 shows the relative location and the size of the deletion-mutants of a p43 prepared in the present invention compared to a normal p43.

Fig. 2. shows the result of the SDS-PAGE analysis of the deletion-mutants of the p43 prepared in the present invention and the p43 protein after purification.

Fig. 3 illustrates the amount of the TNF produced after incubation of human monocyte THP-1 cells to which the purified p43 protein and its deletion-mutants are added respectively.

Fig 4. illustrates the amount of the IL-8 produced after incubation of human monocyte THP-1 cells to which the purified p43 protein and its deletion-mutants are added respectively.

DETAILED DESCRIPTION OF THE INVENTION

The present invention will be described in detail.

According to the present invention, the peptides that have an amino acid sequence - represented by SEQ ID NO:1 to SEQ ID NO:3 as an effective component of an immunological enhancement agent - are the N-terminal domain of the p43 protein. More particularly, these peptides consist of peptide having amino acids from 1 to 147 of the p43 protein, peptide from 1 to 108 of the p43 protein, and peptide from 91 to 256 of the p43 protein.

In order to obtain the peptides that comprise the N-terminal domain of the p43, first, the deletion-mutants of the p43 protein were constructed. Then they were purified and added to cultivated mammalian cells. Next, the degree of inducing the production of cytokine was measured. Finally, the peptides showing a high level of cytokine induction in cultivated mammalian cells could be isolated from various deletion-mutants of the p43 protein.

To indicate the p43 protein or its deletion-mutants, for the sake of convenience, the first and the last number of amino acid sequence of the p43 protein was used in parentheses; for example, the peptide having amino acids from 91 to 256 of the p43 was represented by "p43(91-256)".

In one embodiment of the present invention, recombinant vectors were respectively prepared according to known technique (Park, S. G. *et al.*, *J. Biol. Chem.*, 274:166673-16676, 1999). The recombinant vectors include the following genes respectively encoding: the p43(1-312), which is the p43; the p43(148-312), which is the C-terminal domain of the p43 or the EMAPII; the p43(1-147), the p43(1-108), and the p43(91-256) which include N-terminal part of the p43. The vectors for cloning the above genes are not limited to specific vectors, but pET28a is preferable. The p43 protein and its deletion mutants can be expressed by transforming host cell with the prepared vectors including gene of the p43 or its deletion mutants. The host cell for transformation is not limited specifically, but *E.coli* is preferable.

In another embodiment of the present invention, the cytokine activity of the p43 and its deletion-mutants protein was investigated. The expressed proteins were added to a mammalian cell cultured in serum-free media, and the amount of inducing production of other cytokines was measured. The mammalian cell is not specifically limited if only it is a growth factor dependent cell, however, a human monocyte THP-1 cell is more preferable. Whether or not the above proteins have immunological activity was investigated by

measuring the amount of cytokine produced that can be induced by the p43 or its deletion-mutant proteins. More particularly, among cytokines the amount of the TNF and the IL-8 produced was measured in the present invention. In order to measure the amount of produced cytokines, a well-known method in the art can be used, particularly, in the present invention, ELISA (Enzyme-Linked Immuno Sandwich Assay) was used.

The p43(1-147), the p43(1-108) and the p43(91-256), the peptides represented by SEQ ID NO:1 to SEQ ID NO:3 according to the present invention, showed cytokine activity and induced the production of the TNF and the IL-8. According to the results confirmed in the present invention, the produced amount of the TNF and the IL-8 induced by the above peptides was higher by 2-3 times more than that by EMAP II. The produced amount of the TNF and the IL-8 induced by the peptide represented by SEQ ID NO:1 was higher by 1.1 times more than by the p43 and higher by 2.5-3 times more than by EMAP II.

The immunological enhancement agent according to the present invention that comprises the N-terminal part of the p43 as an effective component is not limited to a specific form, if only it is a feasible form for being administered to human and animals. Also, it can be a form supported with carrier.

As the carrier, at least one of a solid, a liquefied diluent, a filling agent, and other assistant can be used with the ratio of 0.1 ~ 99.5%. The immunological enhancement agent can be administered orally or non-orally. The non-oral administration can be tissue

partial-, hypodermal-, intramuscular-, arterial-, intravenous-, and rectal administration.

The preferable administration form can be prepared in conventional techniques.

Oral administration can be done by using a solid or liquid form; for example, bulk powders, granules, tablets, capsules, syrups, suspensions, etc. If necessary, the unit administration form for oral administration can be microcapsulated. The extension of the activity period and the sustained release of a formulation can be obtained by coating the formulation or inserting its active components to polymer or wax.

The administration amount of the immunological enhancement agent can be determined preferably by taking into consideration for the age of patient, the body weight, a method of administration, any disease and its state, and so on.

The present invention will be described in more detail by the following examples. However, it should be understood that these examples are provided only for the illustration of the present invention but not intended to limit the scope of the invention in any manner.

Example 1

Preparation of recombinant vector including p43(1-147) gene, protein expression, and its purification

The recombinant vector, including the p43(1-147) gene, was prepared by known method in the art (Park, S. G. *et al.*, *J. Biol. Chem.*, 274:16673-16676, 1999). Plasmid

pM388 provided from Dr. Kiyotaka Shiba in Japan Cancer Institute was restricted with *NdeI* and *XhoI* to obtain the p43 gene, and it was used as a template. The PCR was performed using the forward and reverse primer represented by SEQ ID NO:4 and SEQ ID NO:5 respectively, under the condition as follows; 1 min at 95 °C, 1 min at 58 °C, and 1 min at 72 °C. The resulting PCR product was restricted with *EcoRI* and *Sall* and inserted into pET28a (Novagen, Madison, USA) to prepare the recombinant vector.

E.coli BL21(DE3) was transformed with the above recombinant vector including the p43(1-147) gene. The transformed BL21 was cultured in 100ml of LB medium (Luria Broth; 1g NaCl, 1g Bacto-tryptone, 0.5g yeast extracts) and the p43(1-147) gene was expressed as a His-tag fusion protein form. The expressed p43(1-147) protein was purified by using nickel affinity chromatography and mono Q or S ion-exchange chromatography according to known method in the art (Park, S. G. *et al.*, *J. Biol. Chem.*, 274:16673-16676, 1999). To remove lipopolysaccharide, which induces inflammatory response, the purified protein was dialyzed with the pyrogen-free buffer solution (10mM potassium phosphate(pH 6.0), 100mM NaCl) overnight. After dialysis, the protein was loaded to a polymyxin resin(Bio-Rad) that was equilibrated with the same buffer, incubated for 20 minutes and eluted. The concentration of the remaining lipopolysaccharide was measured using the Limulus Amebocyte lysate QCL-1000 kit (Bio Whittacker). As a result, the concentration of the lipopolysaccharide was below 20pg/ml - not able to induce inflammatory response. The purified protein was then subjected to

SDS-PAGE. As shown in Fig. 2, it was confirmed that the p43(1-147) protein having the molecular weight of 21kDa was isolated purely.

Example 2

Preparation of recombinant vector including p43(1-108) gene, protein expression, and its purification

The recombinant vector, including the p43(1-108) gene, was prepared according to the same method described in the Example 1. The recombinant vector pET28a (Novagen) containing the p43(1-312) gene was restricted with *AspI* and *SalI*. The restricted recombinant vector was treated with the klenow fragment to fill up the DNA ends and re-ligated. And then, the recombinant vector, including the p43(1-108) gene, was prepared by treating ligase and making the vector self-ligation. BL21 was transformed with the recombinant vector, including the p43(1-108) gene, according to the same method described in the Example 1. The expressed p43(1-108) protein was purified and analyzed by SDS-PAGE.

As shown in Fig. 2, it was confirmed that the p43(1-108) protein having the molecular weight of 20kDa was isolated purely.

Example 3

Preparation of recombinant vector including p43(91-256) gene, protein expression, and its purification

The recombinant vector, including the p43(91-256) gene, was prepared according to the same method described in the Example 1, except that the PCR was performed using the forward and reverse primer represented by SEQ ID NO:6 and SEQ ID NO:7 respectively, under the condition as follows; 30 sec at 95°C, 30 sec at 50°C, and 40 sec at 72°C. BL21 was transformed with the recombinant vector, including the p43(91-256) gene, according to the same method described in the Example 1. The expressed p43(91-256) protein was purified and analyzed by SDS-PAGE.

As shown in Fig. 2, it was confirmed that the p43(91-256) protein having the molecular weight of 29kDa was isolated purely.

Example 4

Measurement of cytokine activity

To measure a cytokine activity of the deletion-mutant proteins of the p43 that was purified in the Example 1-3, the following experiment was performed.

The human monocyte THP-1 cells (supplied from the ATCC and cultured selecting the sensitive cells to lipopolysaccharide) were inoculated to the RPMI1640 medium containing 10% fetal bovine serum(FBS) and 50 $\mu\text{g}/\text{ml}$ streptomycin and

penicillin, and cultured in 5% CO₂ at 37°C. The cultured cells were washed twice with serum-free RPMI1640, and then 2×10⁶ cells/ml were inoculated into 24-well plate containing 0.5ml of serum-free RPMI1640 medium. The cells were cultured for 2 hours under the same condition, and stimulated for 4 hours by adding 100nM of the proteins purified in the Example 1-3 respectively. The supernatants were collected, and the concentration of the TNF and the IL-8 was measured using the ELISA kit (PharMingen) according to the manufacturer's instructions. The stimulation experiments were repeated twice. The results of the experiments were shown in Fig.3 and Fig.4, respectively.

Comparative example 1

Preparation of recombinant vector including p43(1-312) gene, protein expression, and measurement of cytokine activity

The recombinant vector, including the p43(1-312) gene, was prepared according to the same method described in the Example 1, except that the PCR was performed using the forward and reverse primer represented by SEQ ID NO:8 and SEQ ID NO:9 respectively, and the pM338 as a template, and *Nde*I and *Xho*I were used as restriction enzymes. BL21 was transformed with the recombinant vector, including the p43(1-312) gene, according to the same method described in the Example 1. The expressed p43(1-312) protein was purified and analyzed by SDS-PAGE.

As shown in Fig. 2, it was confirmed that the p43(1-312) protein having the molecular weight of 42kDa was isolated purely.

To measure a cytokine activity of the purified p43(1-312) protein, the each produced amount of the TNF and the IL-8 was measured according to the same method described in the Example 4. The results were shown in Fig. 3 and Fig. 4, respectively.

Comparative example 2

Preparation of recombinant vector including p43(148-312) gene, protein expression, and measurement of cytokine activity

The recombinant vector, including the p43(148-312) gene, encoding EMAP II was prepared according to the same method described in the Example 1, except that the PCR was performed using the forward and reverse primer represented by SEQ ID NO:10 and SEQ ID NO:11 respectively, and pM338 as a template. BL21 was transformed with the recombinant vector, including the p43(148-312) gene, according to the same method described in the Example 1. The expressed p43(148-312) protein was purified and analyzed by SDS-PAGE.

As shown in Fig. 2, it was confirmed that the p43(148-312) protein having the molecular weight of 26kDa was isolated purely.

To measure a cytokine activity of the purified p43(148-312) protein, the each produced amount of the TNF and the IL-8 was measured according to the same method described in the Example 4. The results were shown in Fig. 3 and Fig. 4, respectively.

Observing that the produced amount of the TNF induced by the p43(1-312)(p43 protein), the p43(148-312)(EMAP II) and the deletion-mutants of the p43 protein, i.e., p43(1-147), p43(1-108), and p43(91-256), as shown in Fig. 3, in case of p43(1-132), the produced amount of TNF was 1,500pg/ml, p43(148-312) was 500 pg/ml, p43(1-147) was 1,650 pg/ml, p43(1-108) was 1,000 pg/ml, and p43(91-256) was 2,200 pg/ml. From these results, it was confirmed that the amount of the TNF produced by the p43(1-312)(p43 protein), and proteins comprising the N-terminal part of the p43 protein, i.e., p43(1-147), p43(1-108), and p43(91-256) was much more than that by the p43(148-312)(EMAP II).

Observing that the produced amount of the IL-8 induced by the above proteins, as shown in Fig. 4, in case of p43(1-132), the produced amount of IL-8 was 1,495 pg/ml, p43(148-312) was 650 pg/ml, p43(1-147) was 1,650 pg/ml, p43(1-108) was 1,050 pg/ml, and p43(91-256) was 1,950 pg/ml. From these results, it was confirmed that the amount of the IL-8 produced by p43(1-312)(p43 protein) and proteins comprising the N-terminal part of the p43 protein, i.e., p43(1-147), p43(1-108), and p43(91-256) was much more than that by p43(148-312)(EMAP II).

